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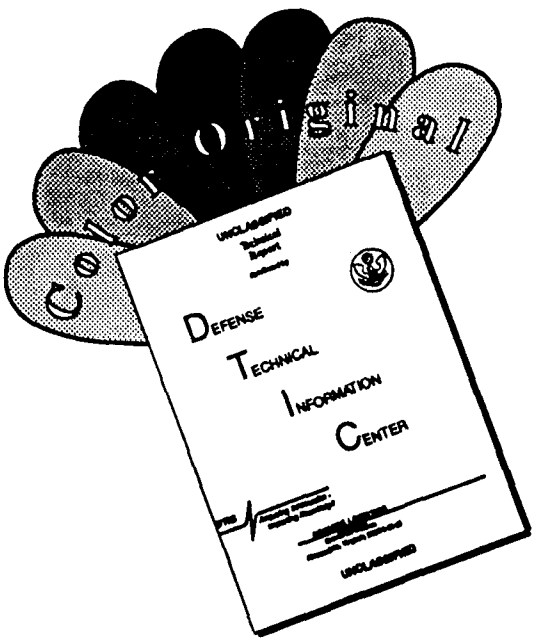

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Progesterone and estrogen are the key steroidal hormones involved in breast development and tumorigenesis. The effects of progesterone and estrogen are mediated through specific intracellular receptors and the status of these receptors in breast tumors has been used as an important prognostic indicator in determining the probability of disease free survival and response to hormonal therapies. The progesterone receptor (PR) is composed of two isoforms, PRA and PRB which have different transactivation functions *in vitro*. This suggests that these receptors are likely to have different physiological roles in breast development and tumorigenesis. To date no in vivo model exists to address this question. The objectives of this proposal are to establish the collective and individual physiological roles of these receptors in breast development and carcinogenesis *in vivo*.

To achieve the above objective, mouse lines will be examined in which the PR status is altered by either a null mutation or selective ablation of the A or B forms of the PR. The physiological analysis of these mutant mouse lines will provide valuable information on the selective contribution of the PRA and PRB to breast development and tumorigenesis *in vivo*. This information will improve prognostic capabilities with regard to analysis of PR status in breast tumors as well as improved treatment strategies.

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Orla M. Connelly 9/27/95

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INTRODUCTION

Problem

Progesterone and estrogen are the main steroid hormones involved in breast development and tumorigenesis and can have both stimulatory and inhibitory effects on carcinogenesis that are both stage and dose dependent. The effects of these hormones are mediated through specific intracellular receptors. However, the specific contribution of these receptors to proliferation, differentiation and tumor growth of mammary tissue remains controversial. The overall objective of this project is to evaluate the physiological role of the progesterone receptor and its individual A and B isoforms in mammary gland development and tumorigenesis. Our approach is to examine the consequences of ablation of the PR A and B proteins on mammary gland physiology and function using PR null mutant mouse models.

Background

Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (1-3). In the case of mammary gland tumorigenesis, the effects of progesterone and estrogen on carcinogenesis can be both stimulatory and inhibitory and are dose and stage dependent (4). These hormonal effects are mediated by specific high affinity intracellular receptor proteins that are members of a superfamily of related transcription factors (5,6). Binding of steroids to these receptors results in the formation of activated receptor dimers that bind to specific enhancer DNA elements located in the promoter regions of hormone-responsive genes (7,8). The activation or repression of these genes represents the manifestation of the hormonal response.

The mammary gland is the site of milk production and secretion, and in females, is a major site of tumorigenesis (9). Mammary gland development occurs during the fetal, post-natal and adult stages of life (10). The development of the mammary gland occurs primarily post-natally and is directed by a complex signal transduction interplay between hormonal (polypeptide and steroid) and growth factor signals. During pregnancy, progesterone and estrogen promote growth and differentiation of normal mammary tissue by regulating ductal branching, alveolar formation (11) and lobuloalveolar development (12). Studies on the ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones have indicated that receptors for estrogen and progesterone (ER and PR respectively) are present in both stromal and epithelial cells, and begin to exert effects on terminal end bud proliferation at 4 and 7 weeks of age, respectively (13). Furthermore, it now appears that epithelial cells, which can express receptors for estrogen and progesterone, are the major sites of primary mammary carcinomas (14).

Although the general consensus on progestin action in the uterus is that progesterone inhibits the proliferative effect of estrogen and acts as a differentiating hormone, this concept cannot be extended to the breast (3). Considerable evidence has accumulated to implicate progesterone in the proliferation of normal mammary epithelium in virgin animals (15) and in the development of the lobular-alveolar structure in mammary glands of pregnant animals (16). Unlike estrogen action, progesterone is a mitogen, not only in the epithelium of the terminal end buds, but also in the ductal epithelium (17). Depending on the time of administration and the dosage used, progestin agonists have been shown to reverse the anti-tumor effects of the anti-

estrogen, tamoxifen, and induce tumor growth (18). The observation that the tumor inhibitory effect of tamoxifen can be reversed by progestin agonists (18) together with the stage and dose dependent carcinogenic activity of progestin agonists (3) suggest that some of the effects of ERs may be mediated by PRs whose expression is known to be induced by estrogen (19). Taken collectively, the above data supports the proliferative effect of progesterone in normal breast development and in contributing to the oncogenic potential of the breast. Conversely, studies using the carcinogen-induced rat mammary tumor model (20) have shown that early pregnancy (21) or the administration of high doses of progesterone and 17β estradiol (22) shortly after the onset of sexual maturity were effective in reducing the susceptibility of the mammary gland to chemical carcinogenesis. Thus, progesterone appears to have both stimulatory and inhibitory effects on mammary gland tumorigenesis that are stage and dose dependent.

From a clinical standpoint, the estrogen and progesterone receptor status of breast tumors is an important prognostic factor in determining the probability of disease free survival and response to hormonal therapy (2,23). Breast tumors that contain functional ERs and PRs have a higher response to hormonal therapy and higher disease free survival probability (2). However, as tumorigenesis progresses, the disease develops to a state that is characterized by a lack of ERs and PRs and a resistance toward hormonal and cytotoxic therapies.

It has been established that PR is composed of two hormone binding forms *in vivo*, termed PR_A and PR_B (24,25). It is thought that the A and B forms arise as a result of either alternate initiation of translation from a single mRNA (26) or by alternate transcription from promoters within the same gene (27). These receptor isoforms differ only in that PR_B contains an additional stretch of amino acids at the amino terminus of the receptor. Previous experiments have shown that these proteins exhibit different promoter specificities for target gene activation (28) while binding to the same enhancer DNA element (29). Remarkably, recent data have implicated a novel repressor function as well as an activator role for PR_A (30). Depending on the promoter and cell context, PR_A was shown to act as a potent transdominant repressor of PR_B-mediated gene transcription. In addition, the repressor function of PR_A was found to influence the activity of other members of this superfamily of transcription factors which included the glucocorticoid, mineralcorticoid and androgen receptors. Intriguingly, recent transient cotransfection experiments have revealed that PR_B when occupied by progestin antagonists can activate transcription (31). Furthermore, this unusual PR_B mediated antagonist transactivation can be dominantly inhibited by the PR_A isoform. This apparent paradoxical stimulatory action of progesterone antagonists via PR_B, if substantiated, would prompt a reevaluation or the potential efficacy of any chemoprevention strategy involving these 'anti-progestins' in the treatment of breast and uterine cancer.

Although, for two decades, the PR has been shown to be composed of two receptor isoforms, the specific physiological role for each of these two PR subtypes in normal breast development, tumor initiation and progression, has yet to be established. However, the existence of both these receptors in different species and tissues, and the elaborate mechanisms regulating their expression suggests that the absolute and relative levels (receptor status) of PR_A and PR_B in a progestin target cell are critical for the correct cellular response to progesterone and its antagonists. The equimolar expression of both forms of the PR in the same cell would allow the possible formation of two homodimers and one heterodimer (A:A, B:B and A:B). The potential existence of three dimeric forms of PR, each having different transcriptional regulatory specificities, would serve to further expand the repertoire of physiological responses to

progesterone. Although breast tissue may contain an overall equimolar ratio of PR_A to PR_B, it is quite possible that different cell types of this tissue, for example epithelial and stromal cells, may have a different ratio which is critical for the normal functioning of these cells. Therefore alterations in the ratio of PR_A to PR_B, would be expected to contribute to an altered susceptibility of these cells to carcinogenesis and have a dramatic effect on the cellular response to progesterone agonists, antagonists, other steroids and growth factors and proto-oncogenes regulated by progesterone.

An additional level of complexity in the involvement of these receptor isoforms in mammary gland development and tumorigenesis arises from influence of growth factors and proto-oncogenes such as epidermal growth factor (EGF), c-myc and cyclin D1 which have been shown to be increased by progestins in cultured human breast cancer cell lines (32). These mitogens may represent "early target" genes for progesterone which may act via autocrine and paracrine mechanisms to influence breast tissue proliferation and differentiation. At this stage, it is not known which of these gene products are modulated by either one or both isotypes of PR.

Purpose of the Present Work

Based on the above observation, we propose the following hypothesis:

During breast development and tumorigenesis, progesterone mediates its mitogenic effect through two receptor isoforms, PR_A and PR_B. We predict that, *in vivo*, PR_A and PR_B have distinct physiological effects and that the ratio of PR_A to PR_B is a key determining factor for normal breast development, oncogenic potential and carcinogenesis.

Methods of Approach

We have used a genetic approach to test the above hypothesis. Two fundamental questions regarding the role of progesterone and its receptor in breast development are being addressed. These are: (1) What is the *in vivo* functional significance of progesterone in general breast development? and (2) What is the *in vivo* functional relevance of the A and B forms of PR in normal breast development and tumorigenesis. These questions will be answered by the physiological analysis of mutant mice deficient in both forms of the receptor (PR_{A+B}-ve) and mouse lines deficient in either the A or B form of the receptor (PR_A-ve and PR_B-ve respectively). The generation of these mouse models will be accomplished by the mutation of the endogenous mouse PR gene by homologous recombination (gene targeting) in mouse embryonic stem (ES) cells. Pluripotent ES cells carrying the mutated PR allele will be injected into mouse blastocysts where they will become the progenitor cells of most of the embryonic tissues including the germ line. Germ line transmission of the mutated PR allele will allow the creation of mouse strains that are heterozygous and homozygous for the mutant PR gene.

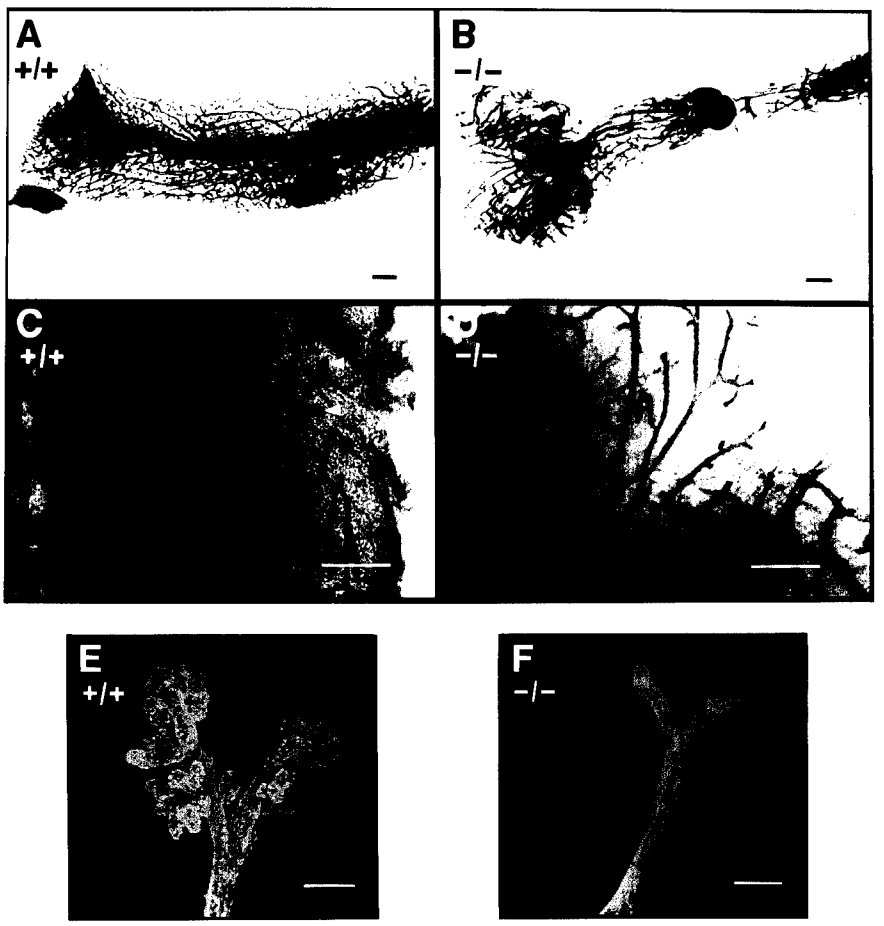
Progress

At the time this project was initiated, we had generated chimeric mice using embryonic stem cells carrying a null mutation of the mouse progesterone receptor gene. During the past year these mice have been bred to produce homozygotes incapable of expressing the

progesterone receptor A and B proteins. At this stage of the project we have completed morphological analysis of the effects of the null mutation on the development of the mammary gland in female animals.

In these studies mammary gland whole mounts were initially prepared from the inguinal mammary gland isolated from intact, hormonally untreated virgin homozygotes and their wild type littermates. Morphological analysis of these glands consistently showed no significant difference in ductal development between the homozygote and wild type mouse virgin glands. In both cases the glands were comprised of mainly an undeveloped ductal morphology.

Next we determined whether the PR mutation had an effect on the development of the mammary gland to the differentiated phenotype that occurs during early pregnancy. Mammary gland whole mounts were taken from ovariectomised 6-week old mice that were hormonally treated for 3 weeks with a daily dose of 1ug of estradiol and 1 mg of progesterone. Following hormonal treatment, hematoxylin staining of whole mounts derived from the wild type mammary gland showed the expected complex ductal arborization to the periphery of the fat pad (Figure 1A and 1C). We have used confocal microscopy to visualize the extent of the lobular alveolar development at the end of the duct (Figure 1E). In contrast to the wild type animals, hormonally treated homozygote females



exhibited a basic ductal structure with limited side branching (Figure 1B and D)and a striking absence of lobuloalveolar development at the end of each duct (Figure1F).

The data obtained in our studies to date provide for the first time unequivocal in vivo evidence that progesterone receptors are required for proliferation of the mammary ductal epithelium as well as in the establishment of the lobular-alveolar system that is required for lactation. Further, in the absence of PR, estrogen induces only a basic ductal architecture, suggesting that some of the previously reported proliferative effects of estrogen are mediated through the PR.

CONCLUSIONS

At this stage of the project we have successfully completed the first of three major goals identified in the original proposal, namely the analysis of mammary tissue phenotype expressed in PR null mutant mice. During the past year we have established the essential role of the progesterone receptor in the proliferation as well as differentiation of mammary epithelial tissue. The demonstration that PR has both a proliferative and differentiative role in the mammary gland emphasizes the importance of establishing the specific role of PR during hormone dependent mammary tumorigenesis and the potential role of antiprogestins in controlling tumor growth. Further, the model provides an excellent system to identify mammary target genes that are specifically regulated by progesterone. During the next phase of the project, we will begin to compare the wild type and mutant mammary glands at a biochemical level to examine the expression of specific target genes involved in proliferation and cell cycle regulation to identify those regulated by PR as detailed in the original proposal. Further, we will continue studies to obtain female mice that selectively express either the PRA or B proteins to identify the individual physiological roles of these proteins in mammary development. Thus, our priorities for the next two years have not changed relative to the original proposal.

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